Supporting Online Material for

Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity

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Materials and Methods

Materials

Antibodies to phospho-Akt S473 (9271, 4058), phospho-Akt T308 (9275), Akt (9272), phospho-p70 S6 kinase (9234), p70 S6 kinase (2708), phospho-S6 ribosomal protein (2215), S6 ribosomal protein (2217), p-4EBP1 S65 (9451), total 4EBP1 (9452), phospho-NDRG1 T346 (3217) mLST8 (3274), mTOR (2972), Raptor (2280) and Rictor (2140) were from Cell Signaling Technology. The phospho-PKCα antibody (sc-12356-R) was from Santa Cruz and the α-Tublin antibody (T5168) was from Sigma. Rapamycin was purchased from Calbiochem (553210) or LC Laboratories. Protease and phosphatase inhibitor cocktail tablets were from Roche (11836153001 and 04906845001, respectively). Tamoxifen was purchased from VWR (IC15673891). Other chemicals were purchased from Sigma unless noted.

Quantitative Real Time RT-PCR Assay

Total RNA was extracted using a Qiagen RNeasy Mini Kit according to manufacturer's instructions. The concentration and purity of RNA were determined by absorbance at 260/280 nm, and 1 μg of RNA was used to generate cDNA using an Invitrogen Superscript III kit with Oligo dT primers. Primers for real-time PCR were obtained from Integrated DNA Technologies. Reactions were run on an Applied Biosystems Prism machine using Sybr Green Master Mix (Applied Biosystems). 36B4, a housekeeping gene, was used to normalize results from gene-specific reactions as previously described (*31*). Primer sequences used to produce gene-specific amplicons are as follows: PEPCK: forward: TGGGTGATGACATTGCCTGG; reverse: ACCTTGCCCTTATGCTCTGCAG (*32*). G6Pase: forward: GAAGGCCAAGAGATGGTGTGA; reverse: TGCAGCTCTTGCGGTACATG (*33*). 36B4: forward: TAAAGACTGGAGACAAGGTG; reverse: GTGTAGTCAGTCTCCACAGA.

Immunoblotting

Cells and tissue samples were lysed in cold RIPA buffer supplemented with phosphatase inhibitor and protease inhibitor cocktail tablets. Cell or tissue lysates were incubated at

4℃ with gentle rocking for 15 minutes or 1 hour, respectively, sonicated on ice for 30 seconds, and then centrifuged at 12,800 rpm for 15 minutes at 4℃; or, alternatively, tissues were lysed in RIPA buffer using a FastPrep 24 (M.P. Biomedicals), incubated on ice for 5 minutes, and then centrifuged. Protein concentration was determined by Bradford or Bicinchoninic Acid (BCA) Assay (Pierce Biotechnology). 20ug protein was separated by sodium dodecylsulpahte-polyacrylamide gel electrophoresis (SDS-PAGE) on 8-16% Tris-Glycine gradient gel or 7.5%, 10%, or 16% resolving gels (Invitrogen). Quantification was performed by densitometry using ImageJ software, and loading was verified by blotting for tubulin or total Akt, as indicated.

Immunoprecipitations

Tissue samples were homogenized in cold 0.3% CHAPS lysis buffer (40 mM Hepes [pH 7.5], 120 mM Nacl, 1 mM EDTA, 0.3% CHAPS, 10mM Pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 0.5 mM Orthovanadate, and protease inhibitors). Cell or tissue lysates were incubated at 4℃ for 15mins or 1 hour, respectively. The cell or tissue lysates were centrifuged at 16,000 rpm for 15 min or 30 min at 4℃ to remove insoluble material. Protein A agarose beads were added to the supernatant and incubated with gentle rocking and rotation for 1 hour. Next, beads were centrifuged out of the lysates and mTOR or rictor antibodies were added to the pre-cleared lysates, which were then incubated overnight at 4℃ with gentle rocking. Protein A agarose beads were added into the supernatant and incubated at 4℃ for a further 1 hour. Immunoprecipitated complexes attached to protein A agarose beads were washed with 0.3% CHAPS lysis buffer three times, boiled in SDS-sample buffer, separated on 7.5% SDS-PAGE, and analyzed by immunoblotting. This method is adapted from that described by Sarbassov (*34*).

Animals and Treatments

All experiments were approved by the Institutional Animal Care and Use Committees at MIT or the University of Pennsylvania, and were performed under the supervision of the Department of Comparative Medicine (MIT) or University Laboratory Animal Resources (Penn). Male C57BL/6 mice were obtained from Taconic at approximately 8 weeks of

age. Raptor floxed mice were generated as described in (*35*) . Rictor floxed mice were generated as described in (*36*). Raptor and Rictor floxed mice were backcrossed to C57BL/6 at least 6 generations. Albumin-Cre mice on the C57BL/6 strain background were obtained from the Koch Institute Transgenic Facility. Ubiquitin C-CreERT2 mice (*37*) were obtained from the Jackson Laboratory (Strain Name: B6;129S-Tg(UBC-cre/ERT2)1Ejb/J, Stock Number: 007001). The genetic background for *mTOR*$^{+/-}$, *raptor*$^{+/-}$, and *mLST8*$^{+/-}$ strains used in lifespan studies was C57BL6/129S5. Chimeric mice were produced by blastocyst injection as previously decribed (*38*). After at least 2 crosses to C57BL/6, colonies were maintained on a mixed C57BL6/129S5 background. Wild-type littermates/cagemates were used for the control groups. *mTOR*$^{+/-}$ *raptor*$^{+/-}$ and *mTOR*$^{+/-}$ *mLST8*$^{+/-}$ strains were generated by crossing the single heterozygotes. Chronic rapamycin treatment was performed by injecting 8 to 10 week old mice intraperitoneally once daily with either 2 mg/kg rapamycin suspended in 0.9% NaCl and 2% ethanol at a concentration of 0.5 mg/mL (547 μM), or vehicle only for 14-28 days. Tamoxifen (IC15673891, VWR) was suspended in corn oil at a concentration of 10 mg/mL, and 200 uL per 25 g of body weight was injected intraperitoneally into 8 to 10 week old male mice once daily for 7 days. Control animals received an equal volume of the tamoxifen suspension, but did not express the CreERT2 fusion protein. We did not observe any influence of the tamoxifen regimen *per se* on subsequent glucose homeostasis, and all mice were allowed to recover for at least one week before use in experiments. Unless otherwise stated, glucose, insulin and pyruvate tolerance tests were performed by fasting the mice overnight for 16 hours and then injecting either glucose (1 g/kg), insulin (0.75 U/kg), or pyruvate (2 g/kg) intraperitoneally. Where indicated, mice were fasted for 6 hours beginning at 8 am to better reflect the conditions of the hyperinsulinemic clamp. Glucose measurements were performed using a Bayer Contour blood glucose meter and test strips, or a Lifescan OneTouch Ultra glucose meter and test strips.

Lifespans and statistics

Lifespans were performed using *mtor*$^{+/-}$, *Raptor*$^{+/-}$, and *mlst8*$^{+/-}$ strains generated as previously described (*38*), as well as *mtor*$^{+/-}$ *Raptor*$^{+/-}$ and *mtor*$^{+/-}$ *mlst8*$^{+/-}$ strains generated by crossing the single heterozygotes. The genetic background was C57BL6/S129, and wild-type littermates of the heterozygous mice were used for the

control groups. Mice were housed with no more than 4 mice per cage. Genotyping was performed as previously described (*38*). The survival of 253 mice was analyzed, with subcategories as follows: Females (30 wild-type, 36 *mtor*$^{+/-}$, 10 *Raptor*$^{+/-}$, 14 *mlst8*$^{+/-}$, 16 *mtor*$^{+/-}$ *Raptor*$^{+/-}$, 17 *mtor*$^{+/-}$ *mlst8*$^{+/-}$); Males (40 wild-type, 32 *mtor*$^{+/-}$, 10 *Raptor*$^{+/-}$, 19 *mlst8*$^{+/-}$, 15 *mtor*$^{+/-}$ *Raptor*$^{+/-}$, 14 *mtor*$^{+/-}$ *mlst8*$^{+/-}$). Mice were fed a diet of RMH 3000 chow diet (Prolab), in MIT's specific pathogen free facility, with minimal disease status during the lifespan study. Survival was calculated using the date each mouse was found dead or was determined to be moribund by veterinary technicians. Survival statistics were calculated using Prism (GraphPad Software) to obtain log-rank values for the lifespan of each strain vs. wild-type, and corrected for multiple comparisons using Dunnett's test with Prism and R. Filling a linear model shows that there is no correlation between date of birth and lifespan, P = 0.9856. A Cox-regression analysis using a likelihood ratio test confirmed that genotype determines lifespan in females (P = 0.019) but not in males (P = 0.97). All other P values in the manuscript were calculated using an unpaired two-tailed Student's t test, except where indicated.

Hyperinsulinemic–euglycemic clamp

An indwelling catheter was inserted in the right internal jugular vein under sodium pentobarbital anesthesia and extended to the right atrium. Four days later, after recovery of presurgery weight and habituation in restrainers, the mice were fasted for 6hours (0700–1300), and administered a bolus injection of 5 μCi of [3–3H] glucose, followed by continuous intravenous infusion at 0.05 μCimin-1. Baseline glucose kinetics were measured for 60 min, followed by hyperinsulinemic clamp for 120 min. A priming dose of regular insulin (16 mUkg-1, Humulin; Eli Lilly, Indianapolis, IN) was given intravenously, followed by continuous infusion at 2.5 mUkg-1min-1. A variable intravenous infusion of 20% glucose was administered to maintain blood glucose levels at 120-140 mg/dL for 90 min. At the end of the clamp, 10 μCi 2-deoxy-D-[1-14C] glucose was injected to estimate glucose uptake. The mice were euthanized, and liver, perigonadal white adipose tissue, and soleus and gastrocnemius muscles were excised, frozen immediately in liquid nitrogen and stored at -80°C for analysis of glucose uptake. Rates of whole body glucose uptake and basal glucose turnover were measured as the ratio of the [3H] glucose infusion rate (d.p.m.) to the specific activity of plasma glucose.

Hepatic glucose production (HGP) during clamp was measured by subtracting the glucose infusion rate (GIR) from the whole body glucose uptake (Rd) (*39*, *40*).

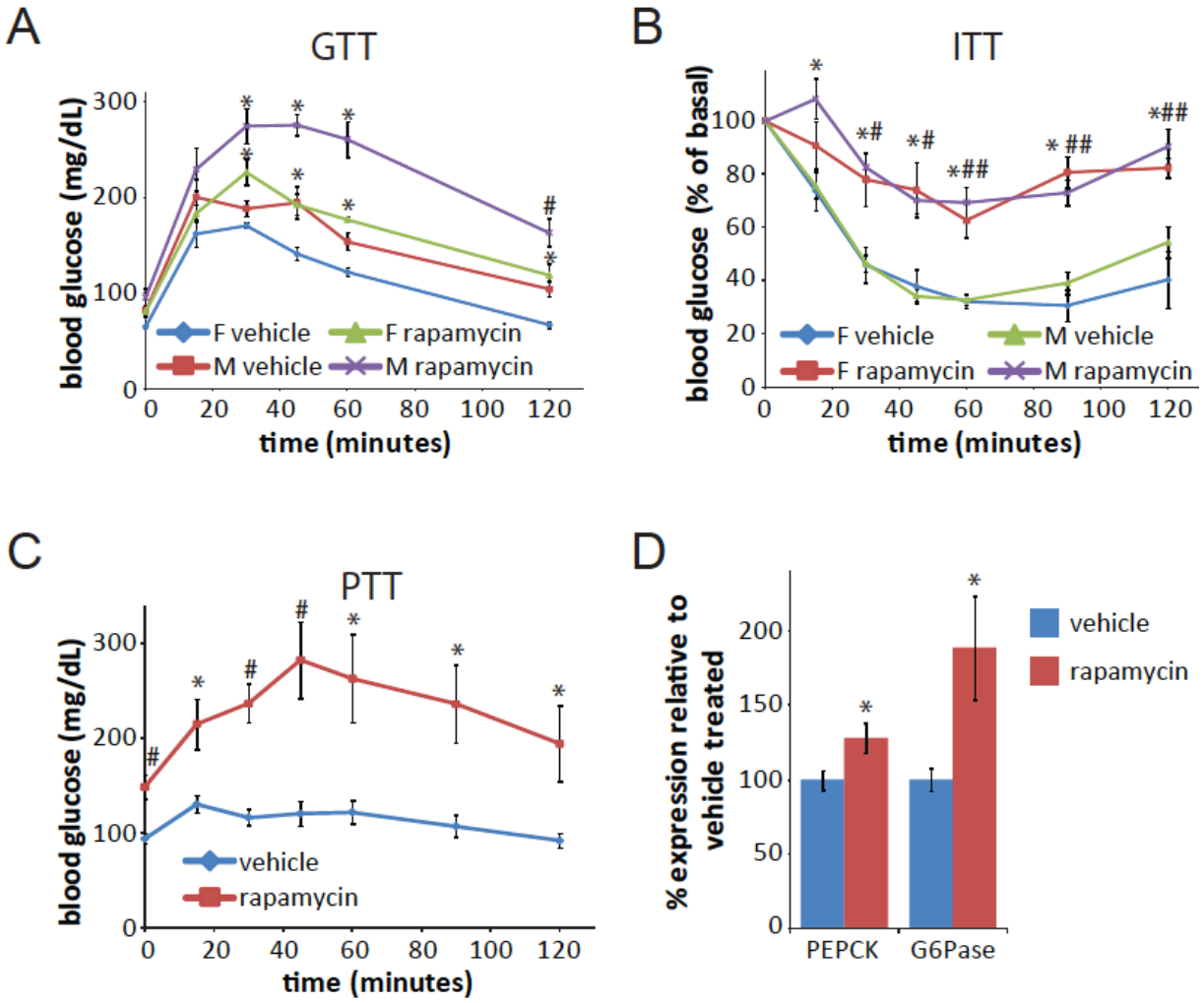


Figure S1. Chronic rapamycin treatment impairs glucose tolerance and insulin sensitivity. Intraperitoneal injection of 2 mg/kg/day rapamycin for two weeks impairs glucose homeostasis as assessed by A) glucose tolerance test (GTT) (* = $P < 0.008$, # = $P < 0.02$) and B) insulin tolerance test (ITT) (* = $P < 0.005$ for males; # = $P < 0.04$, ## = $P < 0.006$ for females) . C) Rapamycin-treated mice are unable to suppress hepatic glucose production, as assessed by pyruvate tolerance test (PTT) (* = $P < 0.05$, # = $P < 0.005$). D) Rapamycin-treated mice have increased hepatic expression of PEPCK and G6Pase (* = $P < 0.05$).

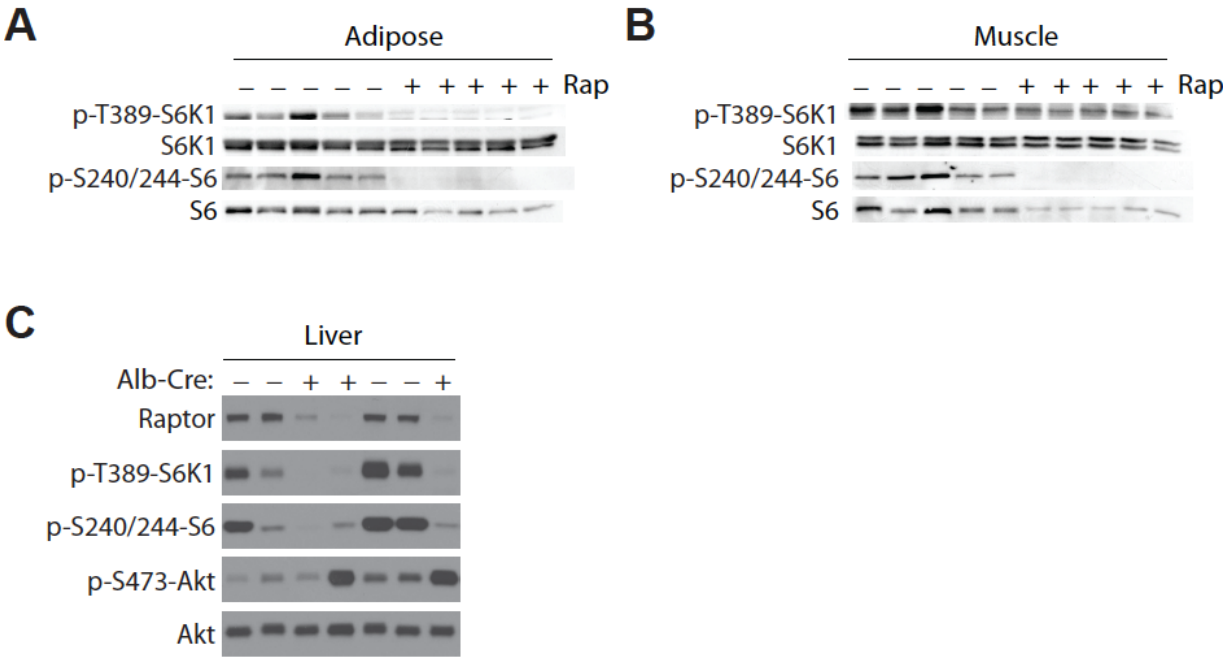


Figure S2. Chronic rapamycin treatment inhibits mTORC1 phosphorylation of S6K1 T389 and subsequent phosphorylation of S6 in (A) white adipose tissue and (B) skeletal muscle. C) Raptor expression was decreased substantially in Alb-Cre *Raptor*$^{loxP/loxP}$ mice.

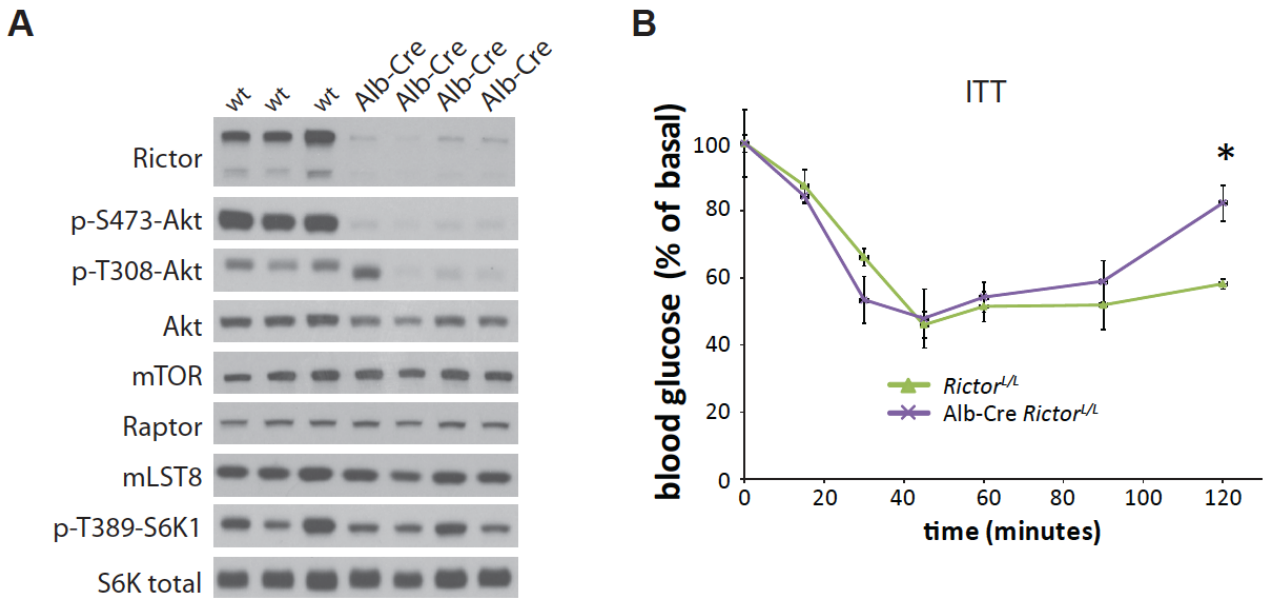


Figure S3. Alb-Cre *Rictor*$^{loxP/loxP}$ mice have A) decreased hepatic Rictor expression and decreased phosphorylation of the mTORC2 target p-Akt S473, and B) nearly normal insulin sensitivity (* = $P < 0.05$).

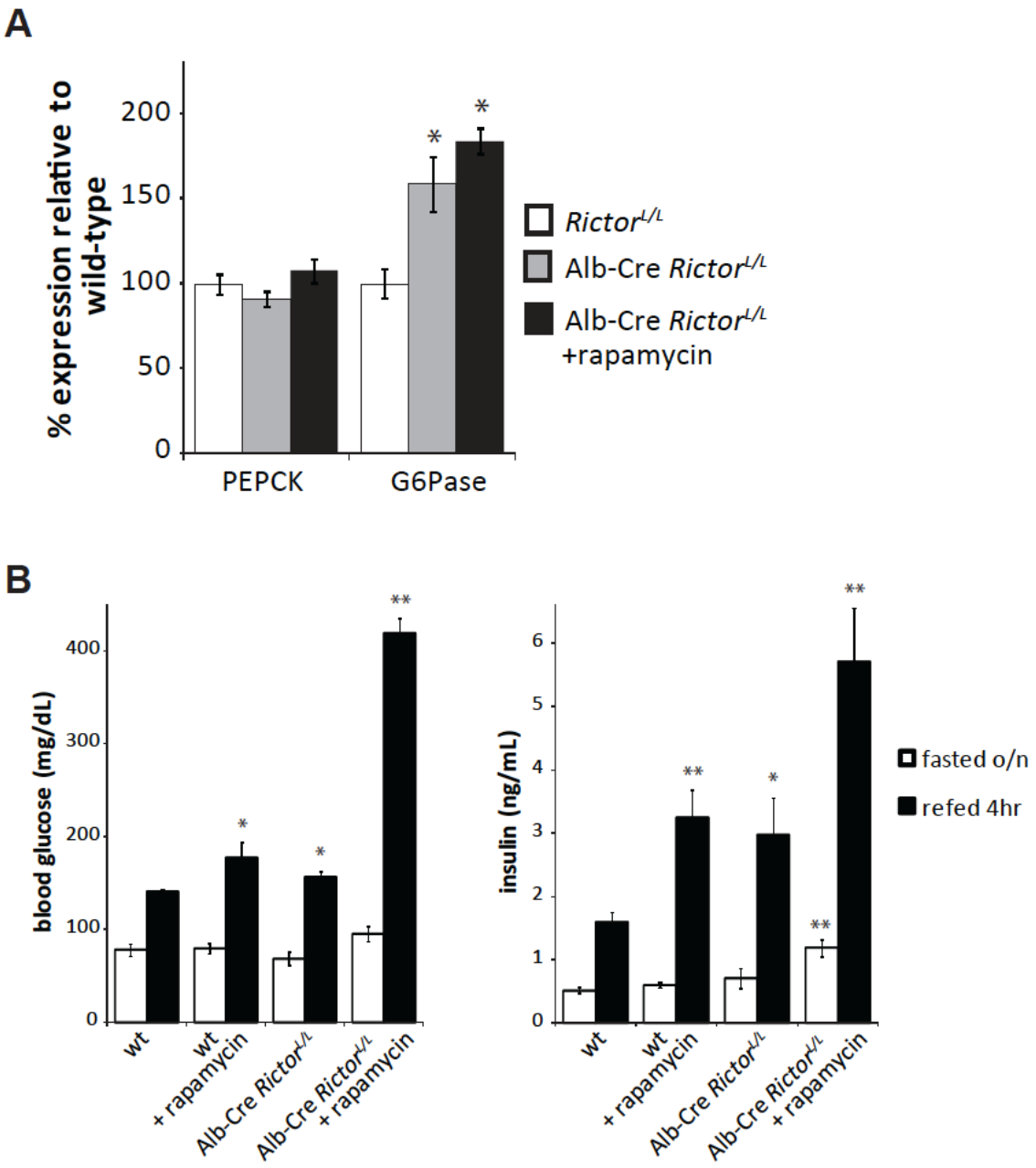


Figure S4. Chronic treatment of Alb-Cre *Rictor*$^{loxP/loxP}$ mice with 2 mg/kg rapamycin causes hyperglycemia, but does not further enhance G6Pase expression or inhibit insulin production. A) Alb-Cre *Rictor*$^{loxP/loxP}$ mice have increased hepatic expression of G6Pase that is not further increased by rapamycin (* = $P < 0.02$ vs. wild-type untreated mice). B) Rapamycin and deletion of hepatic *Rictor* each cause an incremental increase in glucose in mice that have been fasted overnight and then re-fed for 4 hours, and have a strong synergistic effect, leading to pronounced hyperglycemia. These effects are not likely related to β cell dysfunction, as insulin responds appropriately to the increases in blood glucose (* = $P < 0.055$, ** = $P < 0.003$).

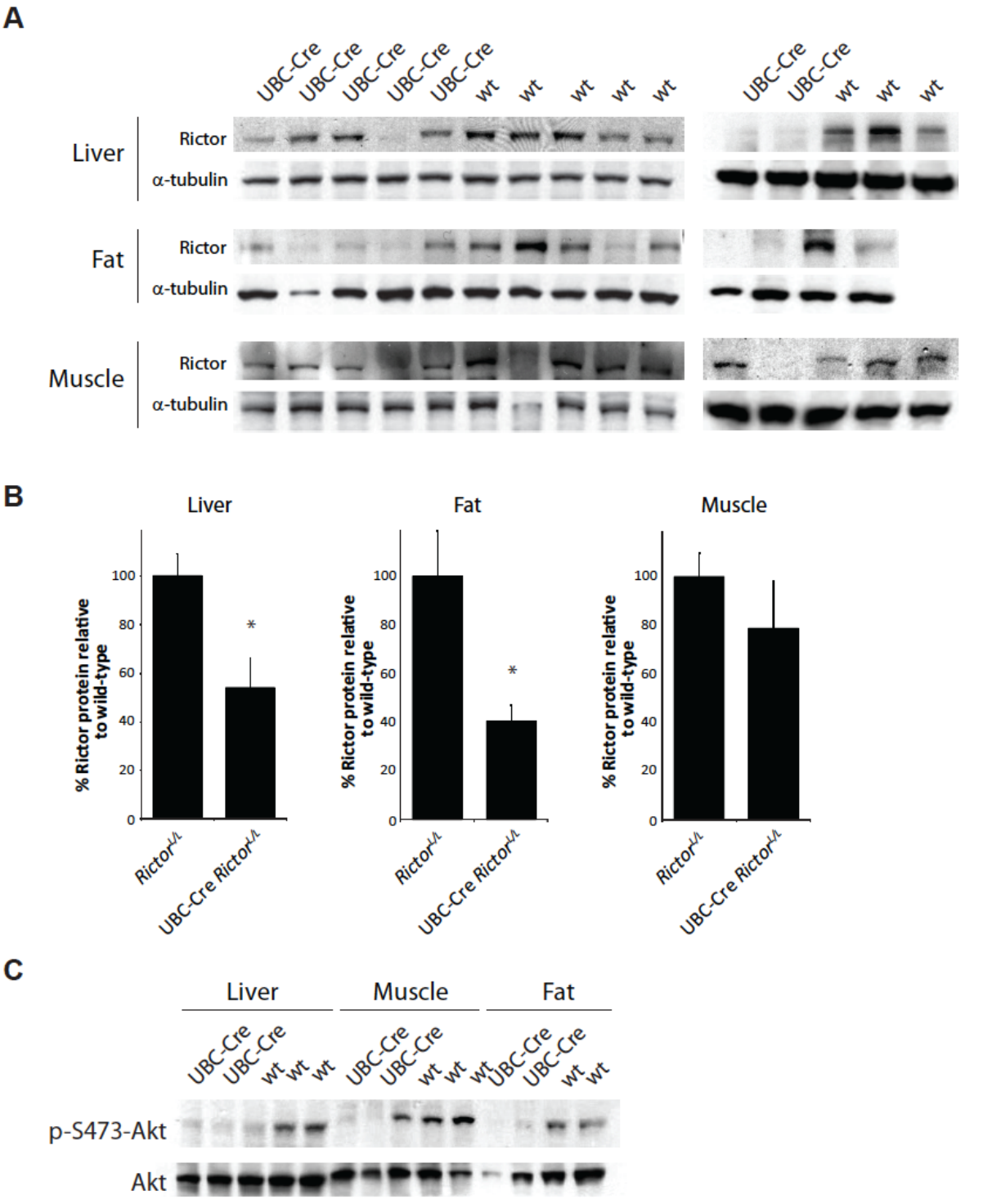


Figure S5. Tamoxifen treatment induces deletion of *Rictor* in UbiquitinC-CreERT2 *Rictor*$^{loxP/loxP}$ mice. A) Rictor expression in tamoxifen-treated Ubiquitin C-CreERT2 *Rictor*$^{loxP/loxP}$ mice used for the hyperinsulinemic-euglycemic clamp study. Tissues were harvested at the completion of the clamp study. B) Quantification of Rictor protein levels in tamoxifen-treated Ubiquitin C-CreERT2 *Rictor*$^{loxP/loxP}$ mice by densitometry, normalized to α-tubulin (* = $P < 0.02$). C) Akt S473 phosphorylation is decreased in tamoxifen-treated Ubiquitin C-CreERT2 *Rictor*$^{loxP/loxP}$ mice.

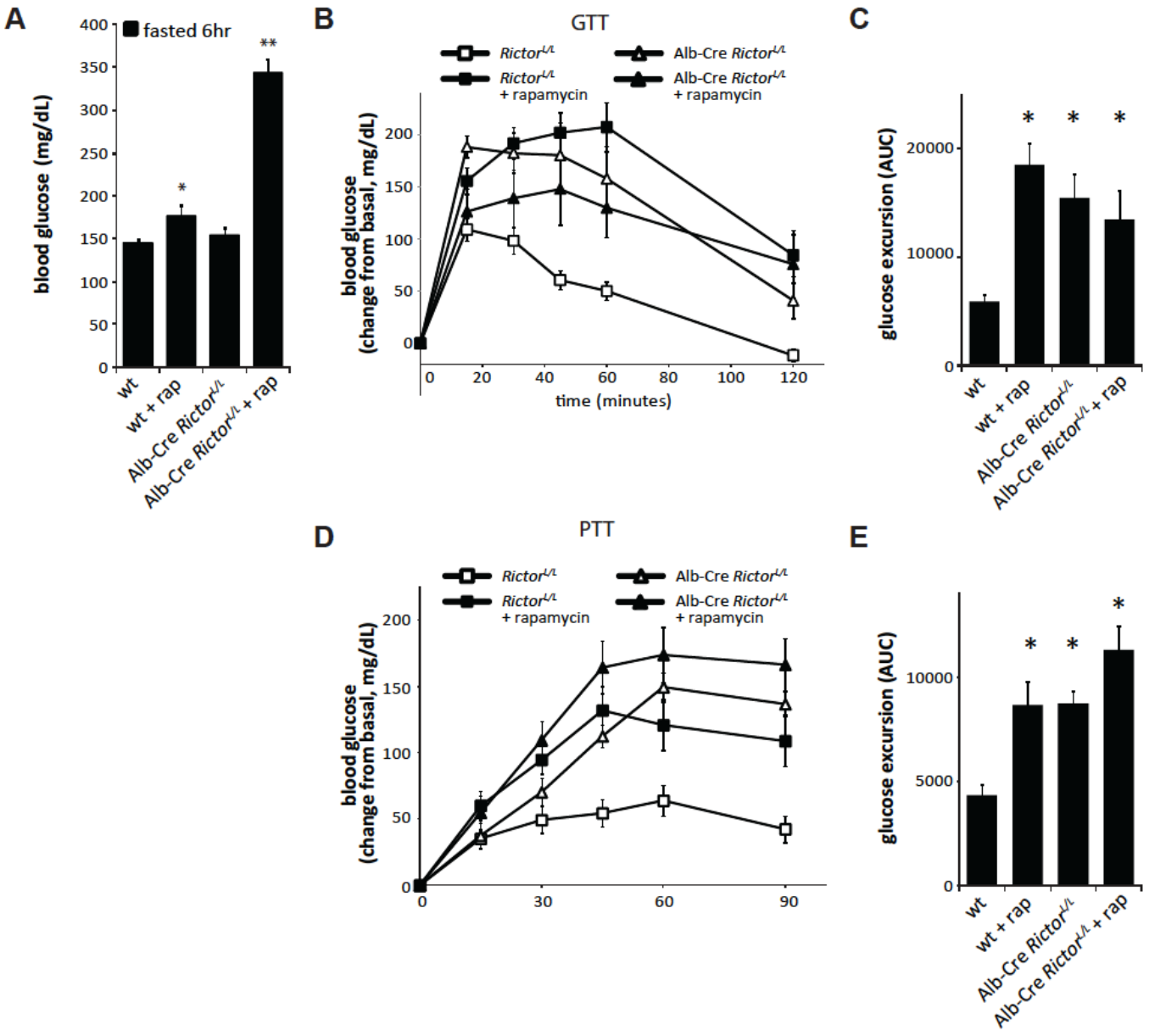


Figure S6. Chronic treatment of Alb-Cre *Rictor*$^{loxP/loxP}$ mice with 2 mg/kg rapamycin causes hyperglycemia, but does not change glucose or insulin tolerance. A) Alb-Cre *Rictor*$^{loxP/loxP}$ mice are hyperglycemic at 6 hours of fasting (* = $P < 0.022$, ** = $P < 0.001$). B, C) Rapamycin and deletion of hepatic *Rictor* do not have additive effects on glucose tolerance after a 6 hour fast (* = $P < 0.001$). D, E) Rapamycin and deletion of hepatic *Rictor* do not have additive effects on pyruvate tolerance (* = $P < 0.003$).

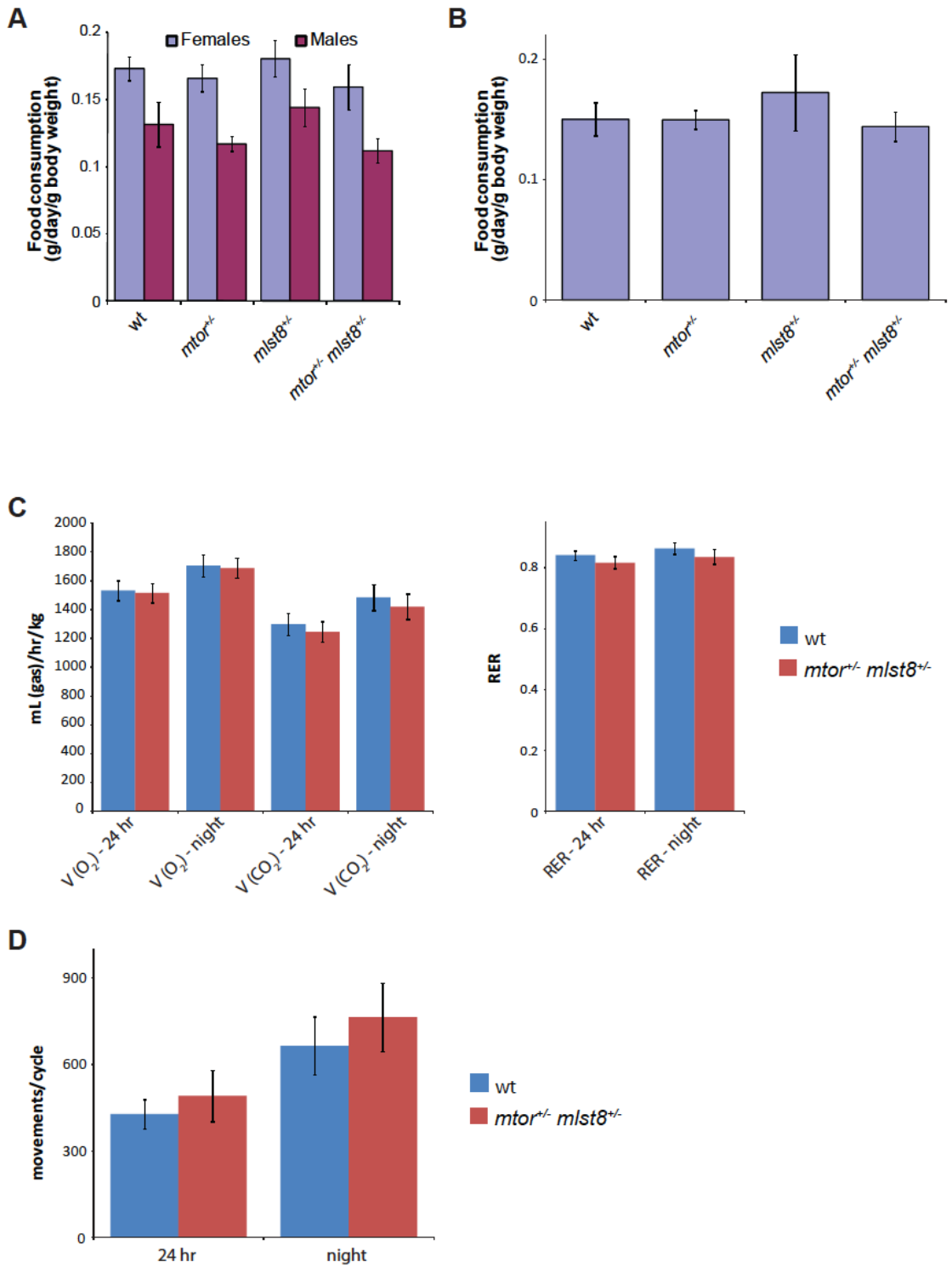
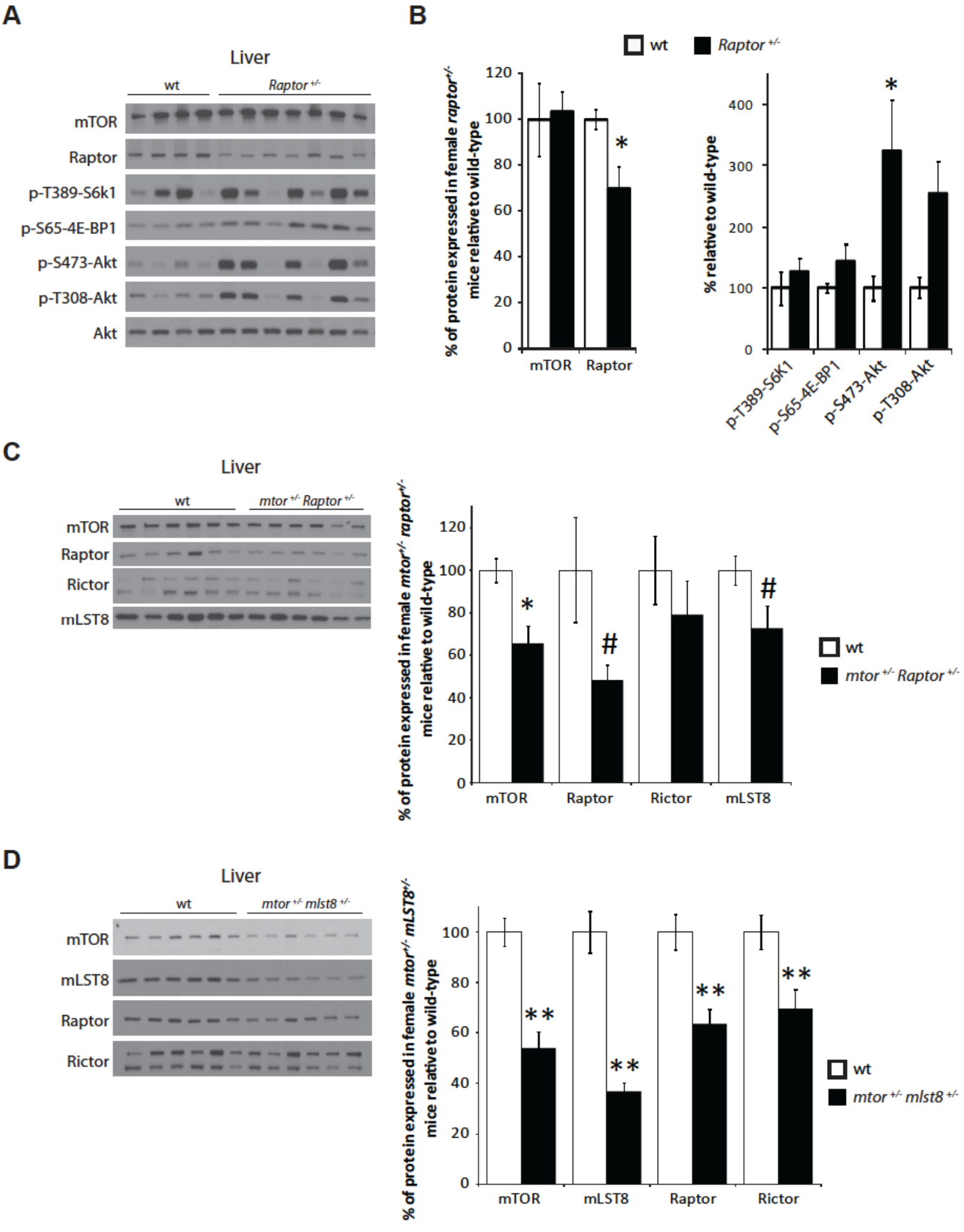


Figure S7. Female *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice are not calorie restricted. A) Young (3 month old) *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice have normal food intake, as do B) 2 year old *mtor*$^{+/-}$ *mlst8*$^{+/-}$ female mice. C) Female *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice have normal metabolism as measured using a metabolic chamber, and D) do not display altered activity.


A
Liver
wt
Raptor +/-
mTOR
Raptor
p-T389-S6k1
p-S65-4E-BP1
p-S473-Akt
p-T308-Akt
Akt
B
wt
Raptor +/-
% of protein expressed in female raptor+/- mice relative to wild-type
mTOR
Raptor
% relative to wild-type
p-T389-S6K1
p-S65-4E-BP1
p-S473-Akt
p-T308-Akt
C
Liver
wt
mtor +/- Raptor +/-
mTOR
Raptor
Rictor
mLST8
% of protein expressed in female mtor+/- raptor+/- mice relative to wild-type
mTOR
Raptor
Rictor
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D
Liver
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mtor +/- mlst8 +/-
mTOR
mLST8
Raptor
Rictor
% of protein expressed in female mtor+/- mLST8+/- mice relative to wild-type
mTOR
mLST8
Raptor
Rictor
wt
mtor +/- mlst8 +/-

Figure S8. A) Female *Raptor*$^{+/-}$ mice do not have significantly altered hepatic protein levels of mTOR, and have a 30% decrease in Raptor expression (* = P < 0.022). B) Female *Raptor*$^{+/-}$ mice have normal mTORC1 signaling, but elevated Akt S473 phosphorylation (* = P <0.05). C) Female *mtor*$^{+/-}$ *Raptor*$^{+/-}$ mice have decreased protein levels of mTOR and Raptor in their livers (* = P < 0.006; # = P < 0.08). D) Female *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice have decreased expression of mTOR, Raptor, mLST8 and Rictor proteins in their livers (** = P < 0.01). Protein was quantified via NIH Image J, with at least 6 mice per group.

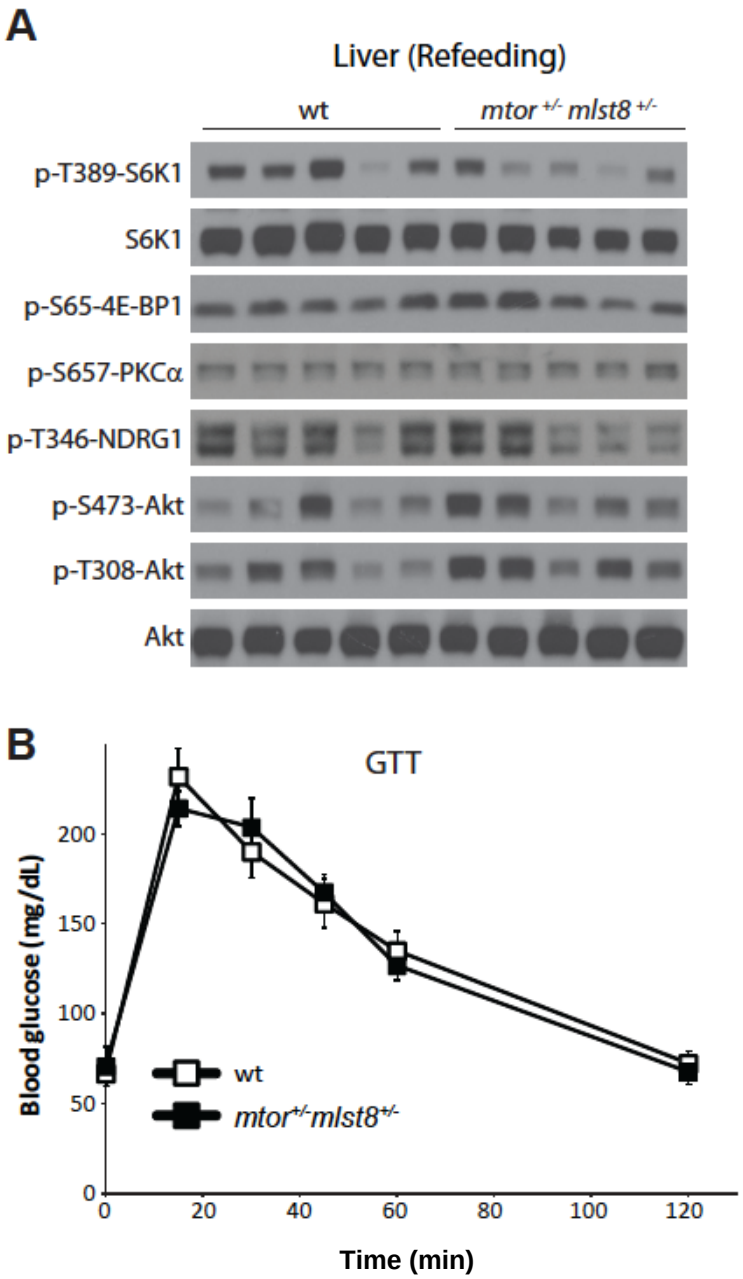


Figure S9. A) Hepatic mTORC1 signaling in *mtor*$^{+/-}$ *mlst8*$^{+/-}$ female mice after an overnight fast and 45 minutes of re-feeding. B) Glucose tolerance test of young female wild-type and *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice.

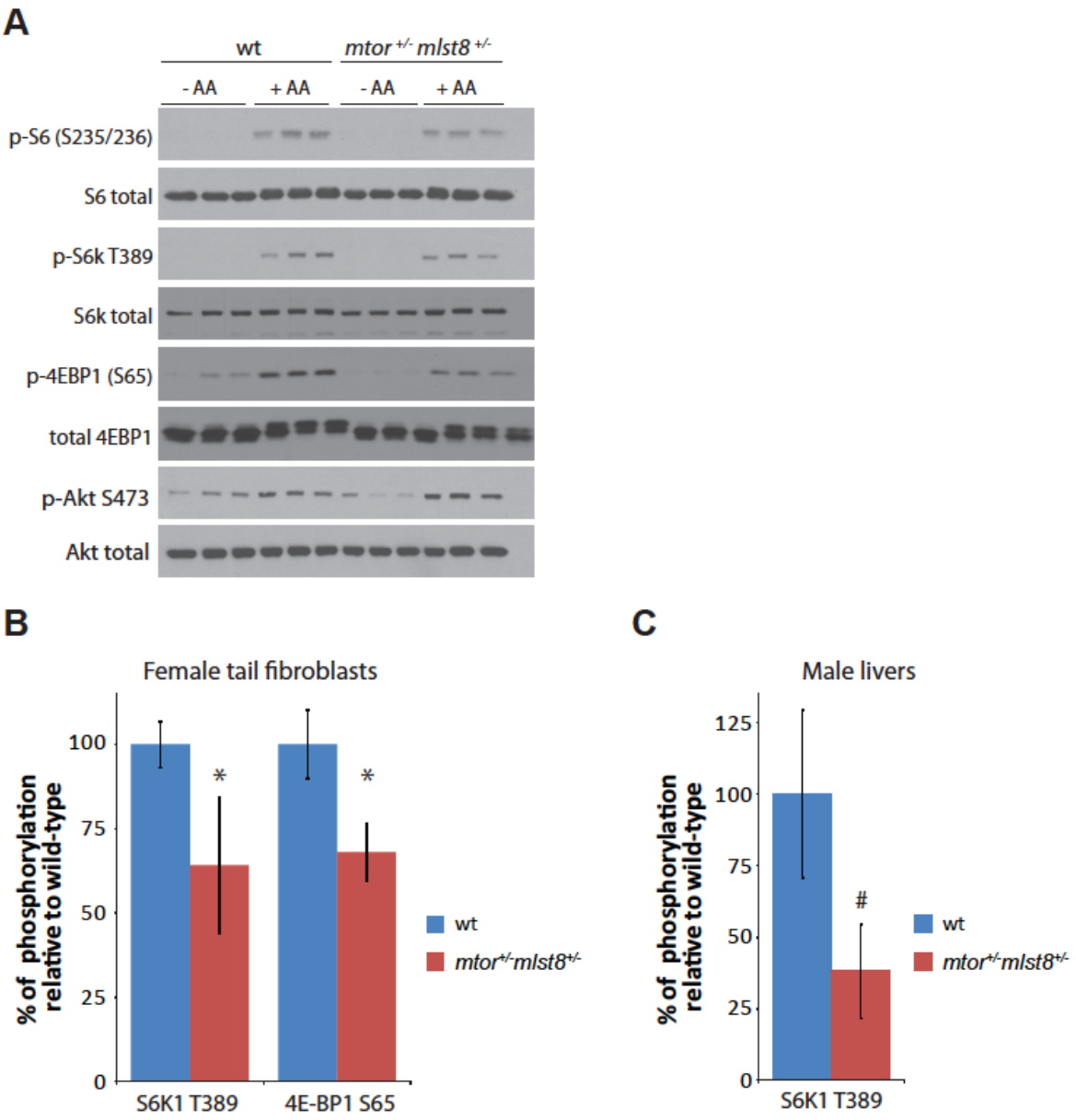
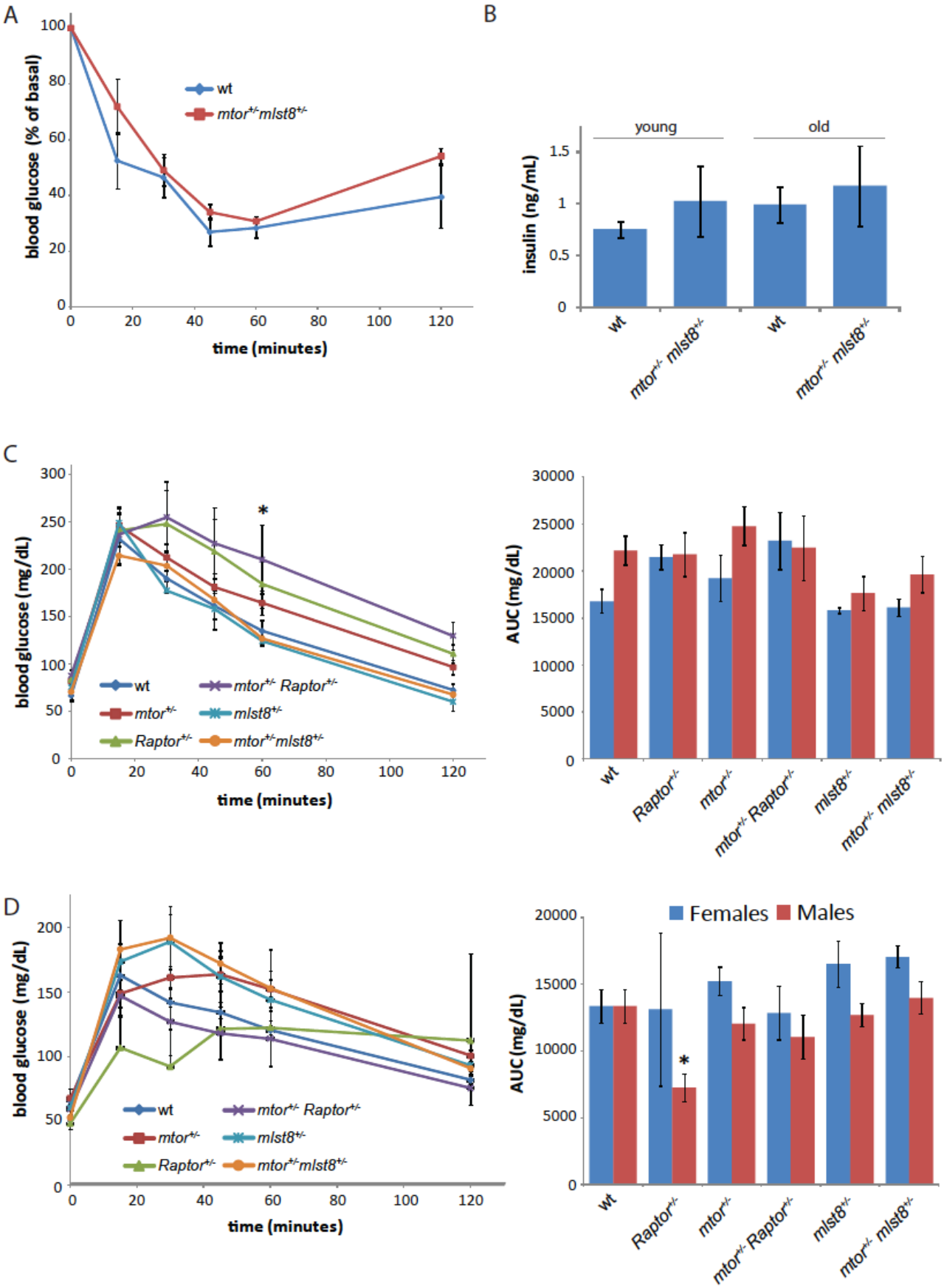


Figure S10. A) Decreased mTORC1 signaling in tail fibroblasts from female wt and *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice. Cells were amino acid deprived for 50 minutes and then stimulated with amino acids for 10 minutes as described previously (*41*). Each lane represents tail fibroblasts independently derived from a different mouse. B) Quantification of phosphorylation (n = 10 wt vs. 10 *mtor*$^{+/-}$ *mlst8*$^{+/-}$ female-derived tail fibroblasts, P < 0.04). C) Quantification of phosphorylated S6K T389 in male mouse livers stimulated by refeeding for 45 minutes (n = 12 wt vs. 12 male *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice, # = P < 0.08).


A
blood glucose (% of basal)
time (minutes)
wt
mtor+/- mlst8+/-
B
insulin (ng/mL)
young
old
wt
mtor+/- mlst8+/-
C
blood glucose (mg/dL)
time (minutes)
wt
mtor+/-
Raptor+/-
mtor+/- Raptor+/-
mlst8+/-
mtor+/- mlst8+/-
*
AUC (mg/dL)
wt
Raptor+/-
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mtor+/- Raptor+/-
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blood glucose (mg/dL)
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mtor+/- Raptor+/-
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mtor+/- mlst8+/-
Females
Males
AUC (mg/dL)
*
wt
Raptor+/-
mtor+/-
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Figure S11. Glucose homeostasis in mice heterozygous for mTOR complex components. A) Female *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice have normal insulin tolerance. B) Fasting insulin levels are not altered in young or old (2 year) female *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice. C, D) Glucose tolerance in young (C) and two year old (D) male and female mice heterozygous for mTOR complex components. Left panels show females only (* = $P < 0.05$, using a two-tailed unpaired t-test vs. wild-type mice and corrected for multiple comparisons).

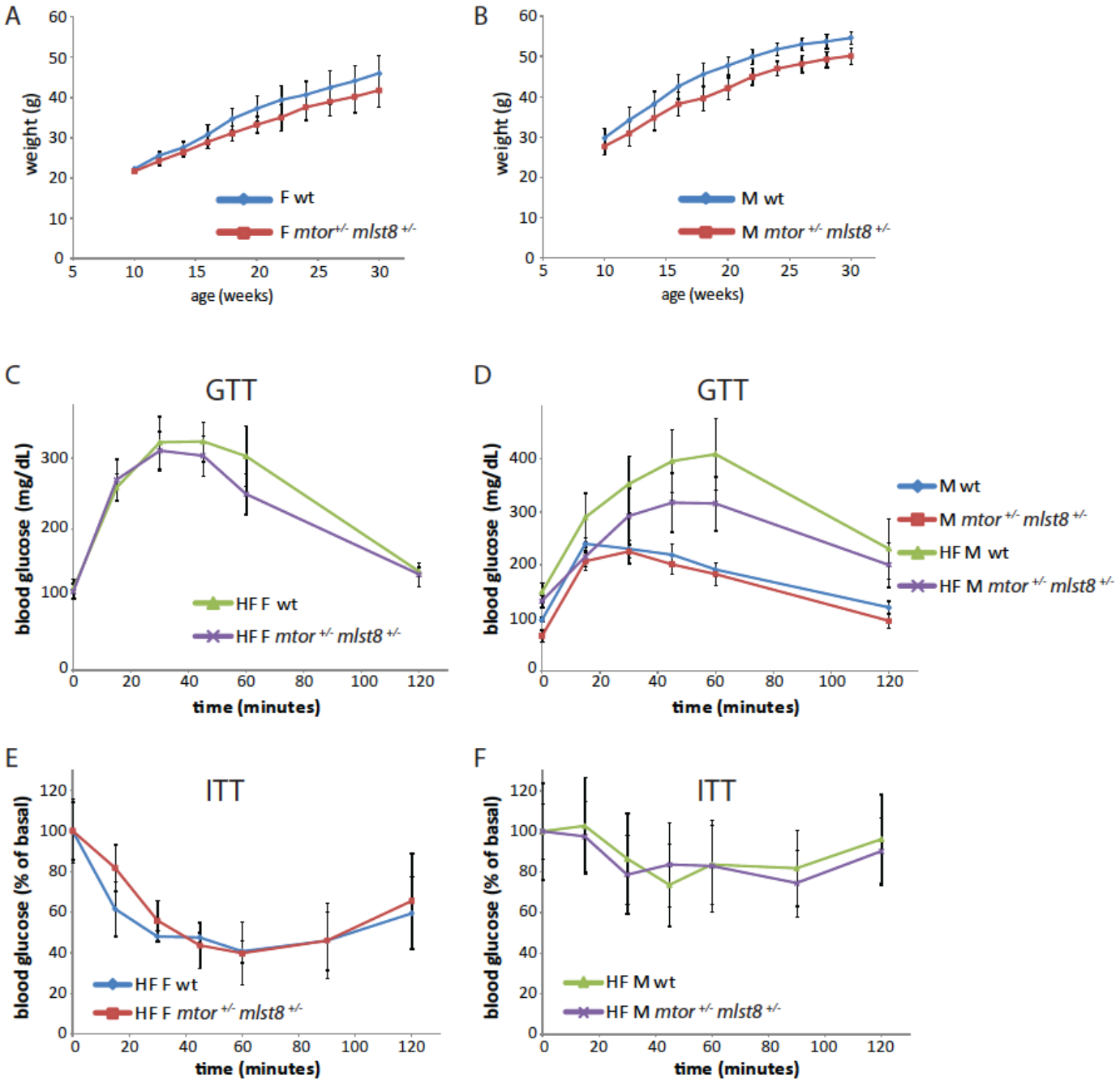

Figure S12. *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice are not resistant to the effects of a high-fat diet. A) Female and B) Male *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice gain weight similarly to wild-type mice when fed a 60% high fat diet for 20 weeks. C) Female and D) Male *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice become glucose intolerant on a high fat diet similarly to wild-type mice. E) Female and F) Male *mtor*$^{+/-}$ *mlst8*$^{+/-}$ mice become as insulin resistant on a high-fat diet as do wild-type mice.

Females		Mean	Median	Max 75%	Max (90%)	P value, Log-rank test	P-value, Dunnett's test	P-value, Cox
	wt	786	786	932	981			
	Raptor$^{+/-}$	752	743	840	869	0.087	0.962	0.127
	mlst8$^{+/-}$	746	724	968	1078	0.657	0.898	0.663
	mtor$^{+/-}$	833	852	1033	1107	0.093	0.574	0.061
	mtor$^{+/-}$ *Raptor*$^{+/-}$	835	843	989	1017	0.224	0.735	0.286
	mtor$^{+/-}$ *mlst8*$^{+/-}$	899	888	1094	1155	0.014	0.046	0.027
Male		Mean	Median	Max 75%	Max (90%)	P value, Log-rank test		
	wt	746	770	907	989			
	Raptor$^{+/-}$	741	748	926	997	0.706	1.000	0.757
	mlst8$^{+/-}$	778	782	900	967	0.675	0.919	0.736
	mtor$^{+/-}$	782	788	928	980	0.326	0.785	0.417
	mtor$^{+/-}$ *Raptor*$^{+/-}$	782	780	940	1026	0.552	0.913	0.471
	mtor$^{+/-}$ *mlst8*$^{+/-}$	737	770	962	1026	0.667	1.000	0.602

Table S1. Lifespan data from mice heterozygous for components of mTORC1. 75% and 90% maximum lifespans are the average lifespan for, respectively, the last 25% or 10% surviving population of each mouse genotype. P values are calculated using the Mantel-Haenszel log-rank test and Prism software for each genotype vs. wild-type, and corrected for multiple comparisons using Dunnett's test with P-values calculated using R. P values for the Cox proportional hazards model were calculated using R.

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